Supplemental Data

"Acetylation of Estrogen Receptor α by p300 at Lysines 266 and 268 Enhances the DNA Binding and Transactivation Activities of the Receptor"

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This file contains the following:

Supplemental Results *(to accompany the Supplemental Figures)*

Acetylation of ERα requires ERα-SRC and SRC-p300 interactions, and correlates with transcriptional activation

The p300-dependent acetylation of ERα occurs under the same conditions that lead to ERα-dependent transcriptional activation (Fig. 1B; (1)). To explore this correlation in more detail, we used two additional approaches: a mutant ERα that is defective in SRC binding (*i.e.*, L540Q) (2, 3) and a set of previously characterized GST-fused fragments of SRC2 that block ERα-SRC and SRC-p300/CBP interactions [GST-SRC2(RID) and GST-SRC2(PID), respectively] (1). In both cases, we measured the (1) acetylation of ERα, (2) targeted acetylation of nucleosomal histones, and (3) ERα-dependent transcription with chromatin templates.

In contrast to wild type ERα, ERα(L540Q) was not acetylated by p300 in the presence of GST-SRC2(RID/PID) and E2 (Supplemental Fig. 1A, *top*). Furthermore, ERα(L540Q) did not direct the acetylation of nucleosomal core histones by p300 in the presence of GST-SRC2(RID/PID) and E2 (*middle*), nor did it support E2-activated transcription with chromatin templates (*bottom*). Likewise, inhibition of ERα-SRC and SRC-p300/CBP interactions by GST-SRC2(RID) and GST-SRC2(PID), respectively, blocked the acetylation of ERα and nucleosomal core histones by p300 in the presence of GST-SRC2(RID/PID) and E2 (Supplemental Fig. 1B, *top* and *middle*, respectively) and inhibited E2-activated transcription with chromatin templates (*bottom*). The effects were not observed with GST-fused SRC2 polypeptides containing mutant versions of the RID and PID (Supplemental Fig. 1B, lanes marked "M"). Collectively, these data indicate that acetylation of ERα requires the recruitment of p300 by SRC to the agonist-bound receptor and correlates with receptor-dependent nucleosomal histone acetylation and transcriptional activation.

Acetylation or mutation of ERα at Lys266/Lys268 does not affect E2 binding, interaction with SRC2(RID), or subcellular localization of ERα

We examined the possible effects of acetylation on ligand binding by ERα (Supplemental Fig. 2A), ERα-SRC2(RID) interactions (Supplemental Fig. 2B), and subcellular localization of ERα (Supplemental Fig. 2C) using a set of Lys266/Lys268 double mutants as described in the text. In [^{3}H]-E2 binding assays with purified receptors, p300-acetylated ERα had a relative ligand binding activity similar to unacetylated ERα (Supplemental Fig. 2A). Likewise, the K266/268R and K266/268Q mutants had relative ligand binding activities similar to wild type ERα (Supplemental Fig. 2A). These results indicate that acetylation of ERα at Lys266/268 does not have an appreciable effect on ligand binding activity.

Next, we used electrophoretic mobility supershift assays to examine the binding of GST-SRC2(RID) by E2- and DNA-bound ERα. Purified wild type or mutant ERα was incubated with a [^{32}P]-labeled double-stranded DNA probe containing an ERE sequence in the presence of E2 and increasing amounts of GST-SRC2(RID). The binding of GST-SRC2(RID) to the E2- and DNA-bound ERα caused a supershift of the complex, as shown in Supplemental Fig. 2B. The amount of the shifted SRC2(RID):ERα:ERE complex gives a relative indication of the extent of the ERα-SRC2(RID) interactions. Both the K266/268R and K266/268Q mutants exhibited relative SRC2(RID) binding activities similar to wild type ERα. The results of the electrophoretic mobility supershift assays suggest that ERα-SRC2(RID) interactions, and perhaps other ERα-coactivator interactions that use a similar interface (*e.g.*, other SRC family

members, TRAP220/DRIP205), are not altered appreciably by acetylation of ERα at Lys266/Lys268. Note that in these assays, the K266/268Q mutant exhibited increased binding to the ERE probe, a result that is addressed in more detail in Fig. 7 in the manuscript.

Subsequently, we examined the subcellular localization of the Lys266/Lys268 mutants by immunofluorescent staining of 293T cells transiently expressing wild type or mutant ERαs. In this assay, wild type ERα was localized primarily to the nuclei, with reduced staining in the nucleoli (Supplemental Fig. 2C), as shown described previously (4). Both Lys266/Lys268 mutants (*i.e.*, K266/268R and K266/268Q) showed a nuclear localization very similar to wild type ERα (Supplemental Fig. 2C), suggesting that the subcellular distribution of ERα is not altered appreciably by acetylation of ERα at Lys266/Lys268. Collectively, these functional assays indicate that acetylation of ERα at Lys266/Lys268 does not affect ligand binding, interaction with SRC2, or subcellular localization of ERα

Supplemental Materials and Methods *(to accompany the Supplemental Figures)*

Synthesis and purification of recombinant proteins

GST-fused SRC2 fragments [*i.e.*, SRC2(RID), SRC2(PID)] were expressed in *E. coli* and purified by glutathione-agarose affinity chromatography as described previously (1). The purified proteins were frozen in aliquots in liquid N_2 and stored at -80°C. Aliquots were analyzed by polyacrylamide-SDS gel electrophoresis with Coomassie brilliant blue R-250 staining relative to BSA mass standards.

In vitro chromatin assembly and transcription assays

In vitro chromatin assembly and transcription reactions were carried out using a reporter template containing four estrogen response elements (EREs) upstream of the adenovirus E4 promoter as described previously (1, 5, 6). Briefly, wild type or L540Q ERα proteins and E2 were added during chromatin assembly, whereas the GST-fused polypeptides [*i.e.*, GST-SRC2(RID) or GST-SRC2(PID)] were added after chromatin assembly was complete. In vitro transcription was performed using HeLa cell nuclear extract as a source of the RNA polymerase II transcription machinery. RNA products from the transcription reactions were analyzed by primer extension (5). The assays were quantified by phosphorimager analysis with ImageQuant v1.2 software (Molecular Dynamics). To ensure reproducibility, all transcription reactions were run in duplicate, and each experiment was performed at least three times.

[^{3}H]-E2 Ligand binding assays

Purified recombinant wild type or K266/268 point mutant ERαs, which were either unmodified, acetylated by p300, or mock acetylated as described above, were used for the ligand binding assays. Twenty nM of the purified receptor protein was incubated with 100 nM [^{3}H]-E2 ("hot") or 100 nM [^{3}H]-E2 + 20 μM unlabeled E2 ("hot + cold") in TEGN buffer (20 mM Tris-HCl pH 7.5, 100 mM NaCl, 0.5 mM EDTA, 10% glycerol) containing 100 μg/ml BSA for five hours on ice in a final reaction volume of 100 μL. After the incubation was complete, 100 μL of a charcoal-dextran mixture was added to each sample with mixing for 5 min to remove the unbound ligands. After centrifugation, 100 μL of the supernatant from each sample was recovered and assayed using a scintillation counter. Specific binding was defined as the counts

in each "hot" sample minus the counts in the corresponding "hot + cold" sample, and were expressed as a percentage of the specific binding obtained with wild type ERα.

Electrophoretic mobility supershift assay

The supershift assays with GST-SRC2(RID) were performed as described in Materials and Methods except that ERα was incubated with the [^{32}P]-end-labeled double-stranded ERE oligonucleotide probe in the presence or absence of purified GST-SRC2(RID).

Immunofluorescent detection of ERα in cells

293T human kidney epithelial cells were grown on poly-L-lysine-treated glass coverslips in 6-well plates in DMEM containing 10% FBS. After 24 hr, at about 70% confluence, the cells were transfected with 600 ng of a vector (pCMV5) for the expression of FLAG-tagged wild type or mutant human ERαs, or the same amount of an empty control vector, using Fugene 6 transfection reagent (Roche Diagnostics Corp., Indianapolis, IN). Sixteen to 20 hr after transfection, the cells were treated with either 10 nM E2 or vehicle for 1 hr, washed in phosphate-buffered saline (PBS), and fixed in 3.7% formaldehyde. The cells then were permeabilized in PBS containing 0.1% Triton X-100 with 10% FBS as a blocking agent to prepare them for incubation with the antibodies. The fixed cells were incubated for 1 hr with primary antibody (1:100 dilution of a custom rabbit anti-human ERα antiserum), washed in PBS, and then incubated for 1 hour with secondary antibody (1:3,300 dilution of goat anti-rabbit IgG conjugated to rhodamine red-X; Jackson ImmunoResearch, West Grove, PA). The nuclei were stained with Hoechst dye during the incubations with the antibodies. The coverslips were mounted face down on glass slides in 50% glycerol/PBS and the edges were sealed with nail polish. The cells were visualized using an Olympus BX-50 epifluorescent microscope equipped with a 100W mercury lamp and were imaged at 60x magnification.

Supplemental References

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Supplemental Table 1. Analysis of ERα acetylation using three different approaches.

Residues covered (+) or not covered (–) by each approach:

Lysine Residue	Deletion Mutation	Site-directed Mutation[1]	Mass Spectrometry[2]	Domain
8	+	+	+	AB
32	+	+	+	AB
48	+	+	–	AB
171	+	+	+	AB
180	+	+	+	DBD
206	+	+	+	DBD
210	+	+	+	DBD
231	+	+	+	DBD
235	+	+	+	DBD
244	+	+	+	DBD
252	+	+	+	DBD
257	+	+	–	DBD
266	+	+	+	DBD
268	+	+	+	DBD
299	+	–	+	LBD
302	+	+	+	LBD
303	+	+	+	LBD
362	+	–	+	LBD
401	+	–	+	LBD
416	+	–	+	LBD
449	+	–	–	LBD
467	+	–	–	LBD
472	+	–	–	LBD
481	+	–	+	LBD
492	+	–	+	LBD
520	+	–	–	LBD
529	+	–	–	LBD
531	+	–	–	LBD
581	+	–	–	LBD

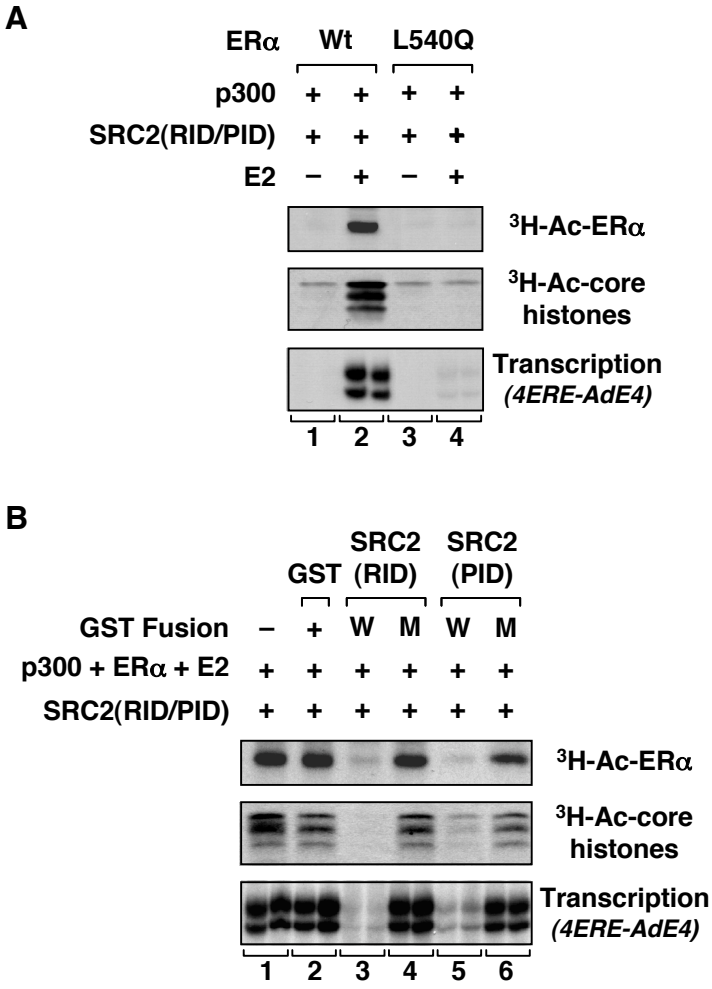
[1]The residues covered by site-directed mutation were based on the results of the deletion mutagenesis analysis. The LBD exhibited no apparent acetylation (Fig. 3C) and the hinge region, as defined in Fig. 3A, contains no lysine residues.

[2]The residues covered by MALDI-QqTOF mass spectrometry represent those residues present in the tryptic peptides that could be observed by the analytical method.

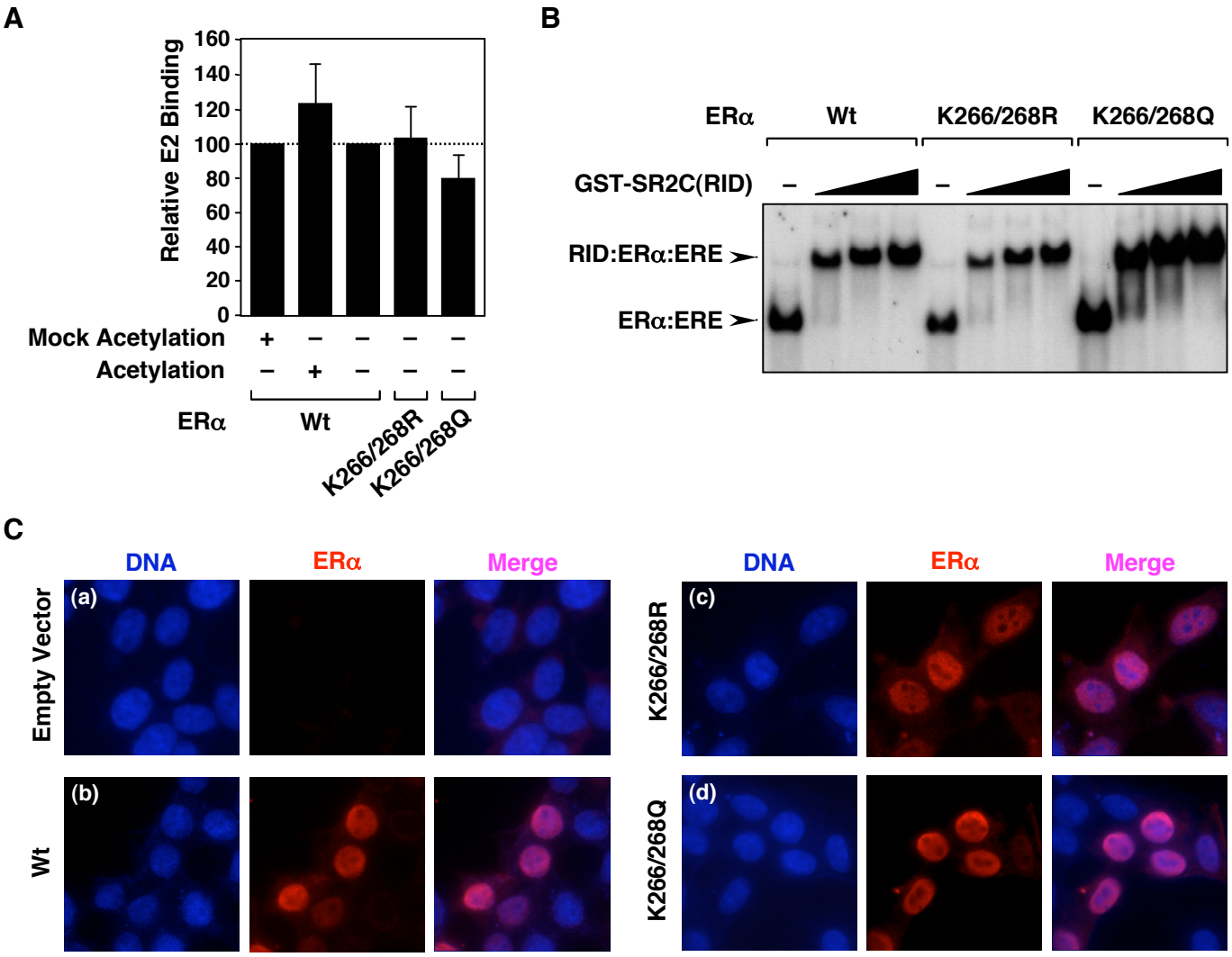
Supplemental Figure Legends *(to accompany the Supplemental Figures)*

Supplemental Fig. 1. Acetylation of ERα requires ERα-SRC and SRC-p300 interactions, and correlates with transcriptional activation.
(A) ERα L540Q, an SRC binding-defective mutant, is not acetylated by p300, does not promote the acetylation of nucleosomal core histones, and is transcriptionally inactive with chromatin templates. Acetylation assays were performed as described for Fig. 1B with wild type and L540Q mutant ERαs *(top and middle panels)*. The transcriptional activities of the wild type and L540Q mutant ERαs were also examined using an in vitro chromatin assembly and transcription assay. Each sample was run in duplicate *(bottom panel)*. Note that p300 and GST-SRC2(RID/PID) were only added to the acetylation assays.
(B) Dominant-negative polypeptide inhibitors that block either ERα-SRC interactions [*i.e.*, GST-SRC2(RID)] or SRC-p300/CBP interactions [*i.e.*, (GST-SRC2(PID)] also block the acetylation of ERα and nucleosomal core histones *(top and middle panels)*, and inhibit ERα-dependent transcription with chromatin templates *(bottom panel)*. GST-fused wild type (W) or binding-defective mutant (M) polypeptides (50-fold molar excess relative to ERα) were added to the acetylation and in vitro transcription assays with chromatin templates as indicated. Note that p300 and GST-SRC2(RID/PID) were only added to the acetylation assays**.**

Supplemental Fig. 2. Acetylation of ERα at Lys266 and Lys268 does not affect E2 binding, interactions with SRC2(RID), or subcellular localization of ERα.
(A) The E2 binding activities of unacetylated (*i.e.*, mock = no acetyl CoA) and acetylated (*i.e.*, + acetyl CoA) wild type ERα, as well as the K266/K268 mutant ERαs (*i.e.*, K266/268R and K266/268Q) were assayed using a [^{3}H]-E2 ligand binding assay. Each bar represents the mean plus the SEM from at least three separate determinations.
(B) SRC2(RID) binding activities of DNA-bound wild type, K266/268R, and K266/268Q ERαs were assayed by EMSAs. The specified ERα proteins were incubated with E2 and a [^{32}P]-labeled double-stranded oligonucleotide containing a single ERE sequence in the presence of increasing amounts of GST-SRC2(RID), as indicated. The reactions were then analyzed by non-denaturing polyacrylamide gel electrophoresis and autoradiography. The shifted and supershifted complexes are indicated by ERα:ERE and RID:ERα:ERE, respectively. Note that the DNA binding activity of the K266/268Q mutant is increased by about five-fold relative to wild type ERα (see Fig. 7C).
(C) Subcellular localization of ERα was examined by immunofluorescent staining of 293T cells transiently transfected with vectors for the expression of wild type or K266/K268 mutant ERαs. The cells were treated with either 10 nM E2 (shown) or vehicle (not shown) for 1 hr before staining for ERα (red) with a rabbit anti-human ERα antiserum (primary antibody) and a goat anti-rabbit IgG conjugated to rhodamine red-X (secondary antibody). The nuclei (blue) were stained with Hoechst dye during the incubations with the antibodies. The merge of the ERα and nuclei staining is shown as fuchsia.



Supplemental Figure 1 - Kim *et al.* (2006)



Supplemental Figure 2 - Kim *et al.* (2006)